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Vigilins Bind to Promiscuously A-to-I-Edited RNAs and Are Involved in the Formation of Heterochromatin

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Summary

The fate of double-stranded RNA (dsRNA) in the cell depends on both its length and location [1]. The expression of dsRNA in the nucleus leads to several distinct consequences. First, the promiscuous deamination of adenosines to inosines by dsRNA-specific adenosine deaminase (ADAR) can lead to the nuclear retention of edited transcripts [2]. Second, dsRNAs might induce heterochromatic gene silencing through an RNAi-related mechanism [3–8]. Is RNA editing also connected to heterochromatin? We report that members of the conserved Vigilin class of proteins have a high affinity for inosine-containing RNAs. In agreement with other work [9–11], we find that these proteins localize to heterochromatin and that mutation or depletion of the *Drosophila* Vigilin, DDP1, leads to altered nuclear morphology and defects in heterochromatin and chromosome segregation. Furthermore, nuclear Vigilin is found in complexes containing not only the editing enzyme ADAR1 but also RNA helicase A and Ku86/70. In the presence of RNA, the Vigilin complex recruits the DNA-PKcs enzyme, which appears to phosphorylate a discrete set of targets, some or all of which are known to participate in chromatin silencing. These results are consistent with a mechanistic link between components of the DNA-repair machinery and RNA-mediated gene silencing.

Results and Discussion

Vigilin and DDP1 Bind Specifically to Inosine-Containing RNAs

We reported previously that nuclear I-RNAs can bind to a complex containing p54^{nrb}, PSF, and matrin 3 [2]. This complex prevents the export of promiscuously edited RNAs to the cytoplasm. Our initial identification of p54^{nrb} and PSF as I-RNA binding proteins came from UV crosslinking studies using HeLa-cell nuclear extracts [2] (Figure 1A). Of special interest, however, is the additional band of about 160 kDa that we observed. Although this band is a minor one in crosslinking studies using HeLa-cell nuclear extracts, in similar studies using whole-cell extracts of *Drosophila* S2 cells, a more striking 160 kDa band specific for I-RNA is observed, along with a number of lower-molecular-weight bands (Figure 1A, lanes 3 and 4). Two of the smaller bands are *Drosophila* homologs of p54^{nrb} and PSF (the NonA and NonA-like proteins) and will be described in more detail elsewhere.

We next used affinity chromatography to isolate HeLa-cell I-RNA binding proteins (Figure 1B). Lane 2 shows the proteins bound to an I-RNA affinity column where about 50% of the guanosines in an RNA transcript have been replaced with inosines. Lane 3 shows proteins that bound to a column containing the same RNA without inosines. We excised prominent bands from the gel and identified proteins by mass spectrometry. As expected, the complex of p54^{nrb}, PSF, and matrin 3 is retained on the column. The 160 kDa band shown here was not consistently observed before, as a result of proteolysis during the course of some experiments.

The 160 kDa band was identified as Vigilin by mass spectrometry. Vigilin is a ubiquitous and abundant RNA binding protein that may play a role in mRNA stability [12–15]. Vigilins are highly conserved (see Table S1 in the Supplemental Data available with this article online), and all members of this family contain 14 tandem hnRNP K-homology (KH) domains (Figure 1D) that could be involved in protein-nucleic acid and protein-protein interactions.

In parallel experiments using *Drosophila* S2 extracts, a prominent 160 kDa protein was also specifically retained by I-RNA (Figure 1C). The *Drosophila* 160 kDa protein was identified by mass spectrometry as the dodeca-satellite binding protein 1 (DDP1), which is the *Drosophila* homolog of Vigilin. Recombinant Vigilin and DDP1 specifically bind to I-RNA and exist in molecular complexes distinct from the other I-RNA binding factors in HeLa cells or S2 cells (Figure S1). DDP1 was originally identified through its ability to bind specifically to one strand of the centromeric dodecasatellite DNA sequence [10]. Thus, this protein binds to both DNA and RNA. In *Drosophila*, DDP1 associates with pericentric heterochromatin and further colocalizes almost everywhere on polytene chromosomes with the heterochromatin-associated protein HP1 [10]. These results suggested a role for DDP1 in both chromosome segregation and heterochromatin formation [10, 16], and this role has recently been experimentally confirmed [11].

Vigilin Is Associated with Heterochromatin in Mammalian Cells

In COS7 cells, Vigilin exhibits a dual localization to both the cytoplasm and the nucleus (Figure S2). In agreement with results reported by others [9], we found that most of the protein is cytoplasmic and associated with ER and that nuclear Vigilin does not appear to reside in discrete, prominent sites but rather is found generally associated with regions of higher DNA content. Like Vigilin, DDP1 is abundant in the cytoplasm of *Drosophila* S2 cells, where it also appears to decorate the ER. However, within the nucleus DDP1 is concentrated in the region of the chromocenter, which appears as a prominent area of DAPI staining (Figure S2). In this work, we have confirmed the reported association of DDP1 with heterochromatin [10, 11] by the observation that DDP1 and HP1 almost completely colocalize.

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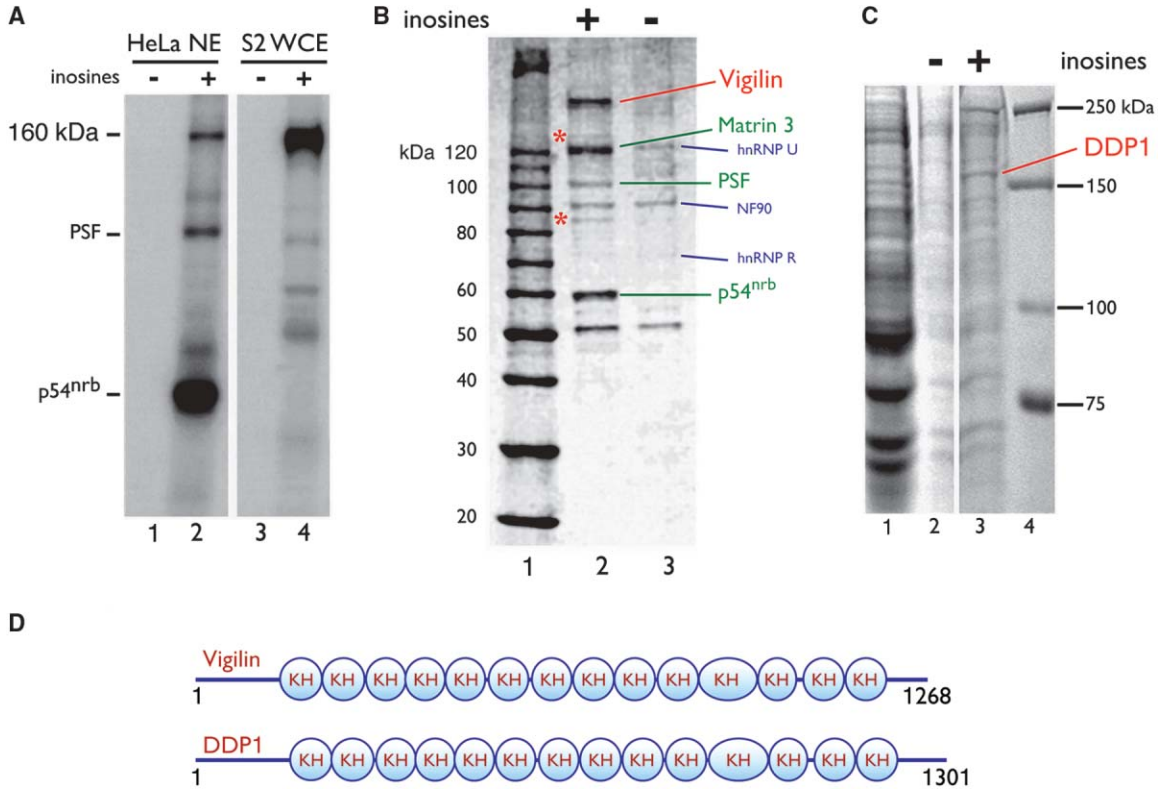

Figure 1. Identification and Isolation of Proteins that Bind Inosine-Containing RNAs

(A) UV crosslinking studies. ^{32}P-labeled RNAs lacking (lanes 1 and 3) or containing (lanes 2 and 4) inosines were mixed with extracts in the presence of an excess of cold yeast RNA competitor and subjected to UV crosslinking [2]. In lanes 1 and 2, HeLa nuclear extracts [2] were used. In lanes 3 and 4, *Drosophila* S2 whole-cell extracts were used. For these extracts, S2 cells were lysed in Dignam Buffer D supplemented with 1% NP-40 and protease inhibitors, then sonicated and clarified by centrifugation.

(B) RNAs containing (lane 2) or lacking (lane 3) inosines were used for affinity selection of nuclear proteins from 300 μl HeLa nuclear extract. Bands were resolved by SDS-PAGE, excised, and identified by mass spectrometry. p54^{nrb}, PSF, and matrin 3 (shown in green) were previously described [2]. Proteins that were isolated from RNA affinity columns regardless of inosine content are shown in blue. The 160 kDa band was identified as Vigilin. The asterisks mark 140 kDa and 86 kDa bands identified as RNA helicase A and Ku86, respectively.

(C) Affinity isolation, as in panel (B), was performed with whole-cell extracts of *Drosophila* S2 cells. The 160 kDa band specifically retained on inosine-RNA-containing columns (lane 3) was identified as DDP1. Lane 1, extract applied to column. Lane 4, markers.

(D) Schematic diagram of the domain structures of Vigilin and DDP1.

In order to demonstrate that Vigilin is associated with heterochromatin in cultured mammalian cells, we performed chromatin immunoprecipitation (ChIP) assays in HEK293T cells transfected with a construct that expresses Flag-tagged Vigilin (Figure 2). We used PCR to examine samples precipitated with the anti-Flag antibodies for the presence of DNA sequences that are known to reside primarily in heterochromatin (the α and β satellite sequences, [17]). Lanes 5 and 12 demonstrate that Vigilin is associated with the heterochromatic α and β satellites, respectively, whereas anti-Flag antibodies do not immunoprecipitate these sequences by themselves (lanes 6 and 13). In control experiments, Vigilin-containing complexes were not enriched for a sequence derived from an actively transcribed gene (lanes 17 and 18).

DDP1 Knockout Affects Nuclear Structure and Chromosome Segregation

Work published to date is consistent with a role of Vigilins in heterochromatic gene silencing (Table S1). In *S. cerevisiae*, the Scp160p protein is, like Vigilin, located primarily on polysomes and the ER [18, 19]. Mutant strains are viable but show defects in chromosome segregation—with enlarged nuclei seen with DAPI staining—and have increased DNA content [18]. The chromosome-segregation defect can be rescued by an overexpression of the *Drosophila* DDP1 protein in yeast cells, strongly suggesting a conservation of function for these proteins [10].

The insertion of a transposable element into the first intron of the DDP1 gene led to a recessive semilethal phenotype [20]. More recently, Huertas et al. [11] reported that mobilization of this element led to virtual elimination of DDP1 expression and function in some strains. DDP1 mutation leads to severe defects in viability and chromosome segregation, as well as to dominant suppression of position-effect variegation (PEV). Furthermore, homozygous-mutant polytene chromosomes show a strong reduction in histone H3 lysine 9 methylation and HP1 deposition at the chromocenter, as judged by immunostaining with specific antibodies [11].

Although we have been unable to strongly reduce Vigilin expression in mammalian cells, Figure 3 shows

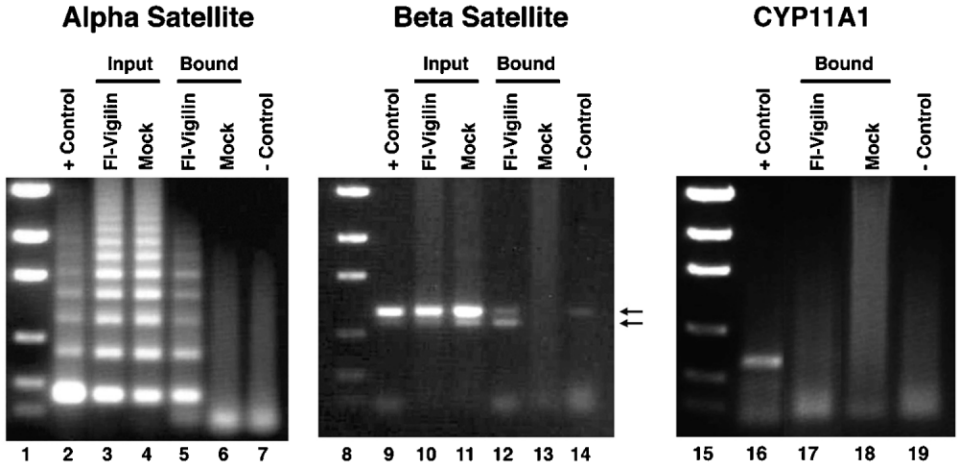

Figure 2. Association of Vigilin with Heterochromatin

An expression plasmid for Flag-epitope-tagged Vigilin (a gift from D. Shapiro) was transfected into HEK293T cells. Forty-eight hours after transfection, the cells were lysed and ChIP assays were performed essentially as described [53], with anti-Flag antibodies to immunoprecipitate complexes containing the recombinant Vigilin. PCR was then carried out with primers specific for α satellite (left panel) or β satellite (right panel) sequences, which are associated with heterochromatic regions of the genome. The α satellite primers (forward primer: 5′-TTTCATTGAG CAGTGTTGAAACACTC-3′; reverse primer: 5′-GTGAGACGAATGTACACAGCAC-3′) generate a ladder of bands with about 170 bp intervals [54]. The β satellite primers (forward primer: 5′-AGGGGCTTTATCCTCATTTCACAA-3′; reverse primer: 5′-GGCCTCCATATTCCCTAACTTCC-3′) generate bands of 450 bp and 520 bp [55]. The arrows on the right indicate the expected positions of amplified β satellite sequences. As a control for a gene not condensed in heterochromatin, we chose P450scc (CYP11A1), the cholesterol side-chain-cleavage gene. Primers for the PCR in lanes 16–19 were 5′-CCAGGCAGAATGTCGGCAGGTGC-3′ and 5′-CGTACACTCCTGCTTCCACCTCTG-3′. Note that this sequence is not enriched in Vigilin-containing complexes. The + controls (lanes 2, 9, and 16) are total cellular DNA subjected to PCR and the − controls (lanes 7, 14, and 19) are PCR in the absence of added DNA.

that we have been able to successfully eliminate DDP1 expression in S2 cells. S2 cells were treated either with double-stranded RNAs (dsRNAs) complementary to DDP1 or with GFP sequence as a control, and additional dsRNA was added every 3 days. Figure 3A shows that elimination of DDP1 expression requires about 12 days of dsRNA treatment. During the course of treatment, the cells exhibited a gradual reduction in growth rate (data

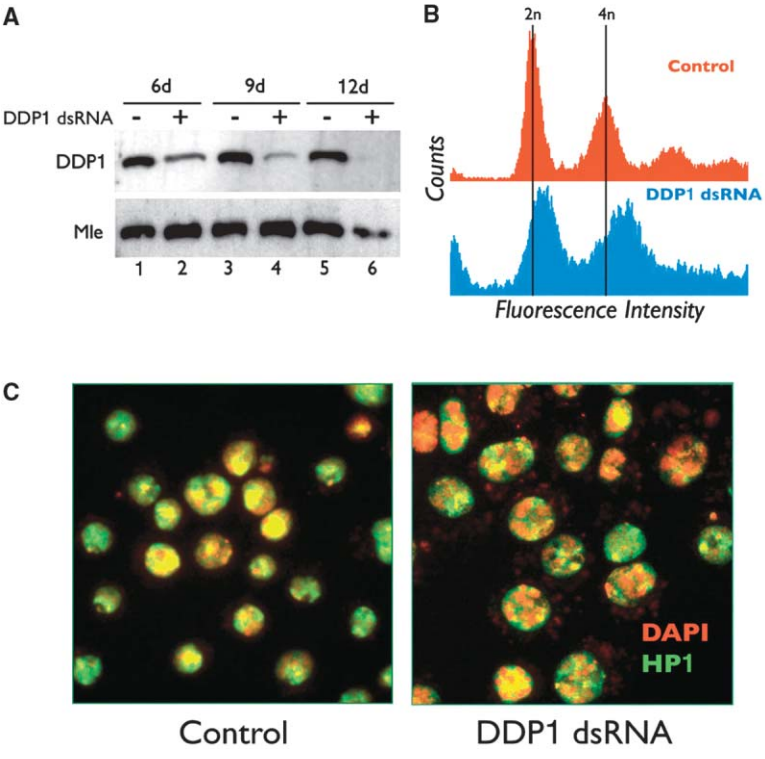

Figure 3. RNAi Knockout of DDP1

(A) dsRNAs complementary to DDP1 or GFP (control) were made and transfected into S2 cells as described [56]. Twenty micrograms of dsRNAs were added to 30 ml suspension S2 culture containing 1×10^6 cells/ml. Additional dsRNA was added every 3 days. Western blotting was carried out at the indicated times, with antibodies to the Mle protein as a control.

(B) FACS analysis. S2 cells (2×10^8) were harvested 24 days after dsRNA treatment, fixed in 70% EtOH, and then treated with 40 μg/ml RNase A before being stained with propidium iodide. FACS analysis was performed with a two-color FACScalibur with argon and red diode lasers (Becton-Dickinson).

(C) Control S2 cells or cells treated for 12 days with DDP1 dsRNA were fixed and stained with DAPI (red) and antibodies to HP1 (green) and photographed at the same magnification.

not shown). After two weeks of dsRNA treatment, the cells were viable but virtually nondividing. In Figure 3B, FACS analysis was used to measure DNA content in control S2 cells and DDP1-depleted cells. The DDP1 knockout cells contained, on average, a higher DNA content than the control cells, and the peaks were broader, suggesting heterogeneity in cellular chromosome or DNA content. Microscopic observation revealed that the nuclei were enlarged and that there was an apparent mislocalization of HP1 (Figure 3C); in DDP1 knockout cells, there was no longer a clear chromocenter. These results are consistent with the recently reported phenotypes of DDP1-knockout *Drosophila* strains [11] and are essentially identical to those reported for Scp160p-null yeast cells [10, 18].

Nuclear Vigilin Is in a Complex with RNA-Dependent Protein Kinase Activity

The finding that both Vigilin and DDP1 interact with inosine-containing RNAs suggests that in higher eukaryotes dsRNA-directed RNA editing may be linked to at least some nuclear heterochromatin. We therefore sought Vigilin partners for clues as to how Vigilin might act. In our original I-RNA affinity-chromatography experiments, we noted two additional bands that correlate with the presence of Vigilin (Figure 1B, asterisks in lane 2). These proteins do not bind directly to I-RNA; we have been unable to see bands of these sizes in UV crosslinking experiments. The 86 kDa band is Ku86. Ku86 exists in cells as a stable heterodimer with Ku70, and this complex has been reported to play a number of important roles in DNA repair and telomere maintenance [21]. In the presence of double-stranded DNA (dsDNA), the Ku heterodimer can recruit the catalytic subunit of the DNA-dependent protein kinase, DNA-PKcs. Figure 4A confirms that Vigilin interacts with the Ku complex. The association of both Ku70 and Ku86 with Vigilin was revealed by Western blotting with specific antibodies (lane 4). Importantly, not only does the nuclear Vigilin complex also contain DNA-PKcs (Figure 4A, lane 4), but this association is also dependent on the presence of RNA. RNase A treatment of the Vigilin immunoprecipitate releases DNA-PKcs but not the Ku70/86 heterodimer (lane 8).

The second Vigilin-associated protein released from the I-RNA affinity column is RNA helicase A (RHA; 140 kDa). Figure 4B shows that RHA, like the Ku autoantigen and DNA-PKcs, is indeed associated with Vigilin in HeLa-cell extracts, and Figure 4C provides an independent demonstration that RHA interacts with the Ku 70/86 heterodimer. As with Ku, the association of RHA with Vigilin does not depend on RNA (data not shown). RHA belongs to a conserved class of RNA helicases and unwinds both dsRNA and DNA [22–25]. It has been reported to have both cytoplasmic and nuclear functions and to be involved in a number of steps of cellular DNA and RNA metabolism, including having effects on chromatin [26]. In the cytoplasm, RHA is polysome associated and may affect mRNA metabolism. In the nucleus, RHA mediates a number of interactions with chromatin bound proteins [26]. Our data, suggesting that RHA is in a complex that can affect chromatin structure, are consistent with the report that a fragment of RHA can inhibit BRCA1 function and lead to defects in ploidy [27]. Figure 4B also shows that ADAR1 associates with this same complex. This important result allows us to connect editing directly to machinery that may have effects on chromatin.

Because the Vigilin complex contains the DNA-PKcs enzyme (Figure 4A), we asked whether it also has kinase activity. Figure 4D shows that this is the case. When Vigilin-containing immunoprecipitates are incubated with γ-^{32}P-ATP, a discrete set of polypeptides becomes phosphorylated. Most prominent among these are proteins with apparent molecular weights of about 17 kDa, 30 kDa, 40 kDa, and 140 kDa (lane 4). As expected, RNase treatment eliminated all kinase activity (lane 8). These results are consistent with DNA-PKcs being responsible for the phosphorylation we see, but it remains possible that a kinase other than DNA-PKcs is also present and active in the Vigilin complex.

Although the kinase activity we observe is RNA dependent, we cannot conclude that only edited RNAs serve as triggers. I-RNAs may represent only a subset of RNAs that act through the Vigilin pathway. Because KH domains are thought to be nucleic-acid binding modules, Vigilins have the potential to interact with a multitude of targets with different recognition sequences. Vigilins may interact with RNAs either by recognizing specific sequences (through their individual KH domains) or by selecting targets based on RNA structure. Some of the original work on the RNA binding properties of human Vigilin indicated that maximal binding requires at least 75–100 nt of RNA and that one defining feature of this RNA is that it is largely "unstructured" [13]. Also, the DDP1 protein was originally isolated as a single-stranded DNA binding protein that specifically binds the "unstructured" dodeca-satellite C-strand of *Drosophila* pericentric heterochromatin [10]. DDP1 binding was also found to be strongly influenced by the length and extent of the secondary structure of the ssDNA substrate. In addition, DDP1 showed a significant affinity for the unstructured pyrimidine-rich strand of the most abundant centromeric *Drosophila* AAGAG satellite [16]. Importantly, I-RNAs are likewise relatively unstructured [28]. Therefore, it is quite possible that the observed interactions are structure based. In this view, Vigilins would mediate gene silencing in response to many triggers, with the common feature being low secondary structure. However, edited RNAs could well represent an important subset of these triggers.

Identification of Phosphorylation Targets

What are the targets of phosphorylation in the Vigilin complex? It has recently been reported that RHA is phosphorylated in an RNA-dependent manner by DNA-PKcs [29]. Thus, we think that it is quite likely that the prominent phosphorylated band at 140 kDa in Figure 4B, lane 4 is in fact RHA. The biological significance of this phosphorylation is still unclear at this point, but our results suggest the possibility that the function of RHA might be modulated by phosphorylation.

We next asked whether specific proteins involved in known Ku functions might be targets of phosphorylation

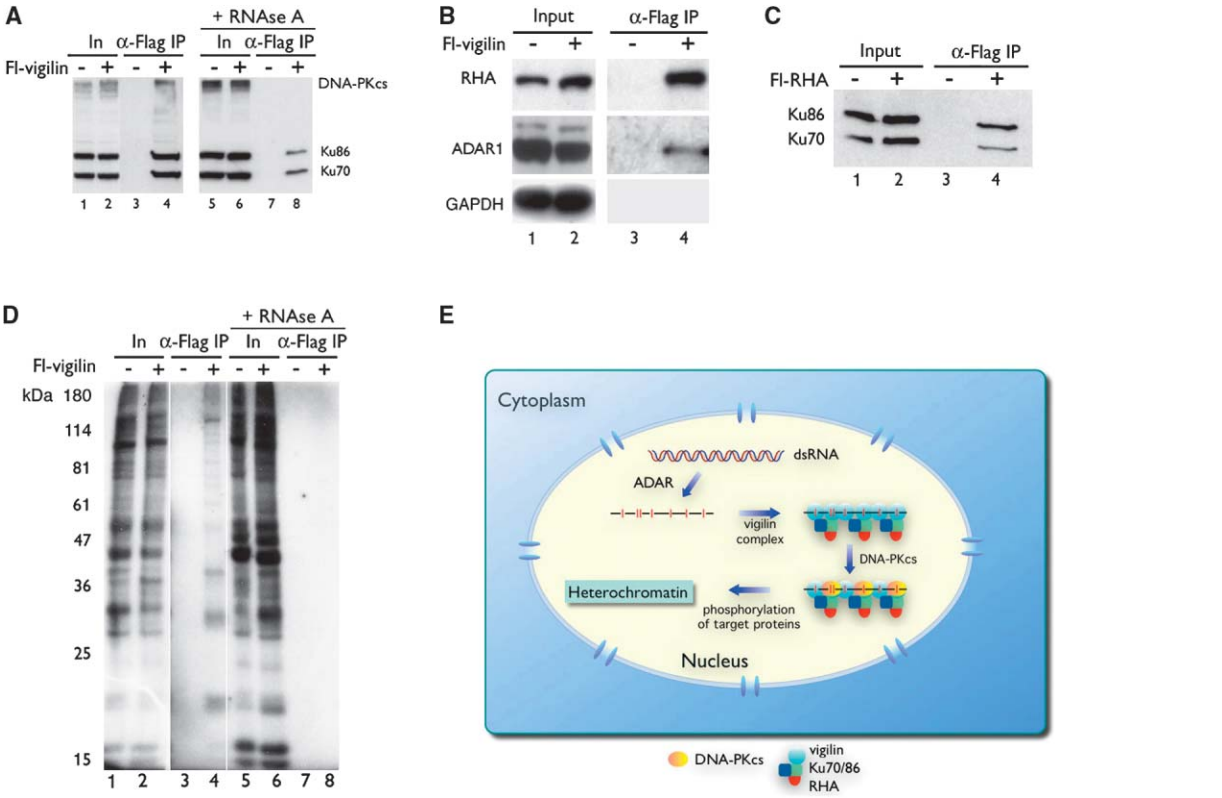

Figure 4. Protein-Protein Interactions

(A) Vigilin is in a complex with Ku86, Ku70, and DNA-PKcs. Seventy-two hours after transfection with or without Flag-tagged Vigilin cDNA, HEK293T cells were harvested and lysed in 300 μl cold hypotonic buffer and then incubated on ice for 20 min. Lysates were clarified by high-speed centrifugation. For immunoprecipitation, 1 mg extract was incubated with 50 μl anti-Flag M2-conjugated agarose beads (Sigma) in 500 μl binding buffer at 4°C for 2 hr. The beads were washed six times with binding buffer, and bound proteins were eluted with SDS. After SDS-PAGE, Western blotting was performed with a mixture of antibodies specific for Ku70, Ku86, and DNA-PKcs. Lanes 1, 2, 5, and 6 show 5 μg aliquots of extracts. In lanes 1, 3, 5 and 7, mock-transfected cells were used. For the experiment in lanes 5–8, the extracts were treated with RNase A before immunoprecipitation. For RNase pre-treatment, 200 μg/ml RNase A was added to cell extracts, and incubation was at 4°C for 2 hr.

(B) Vigilin interacts with ADAR1 and RNA helicase A. Flag-Vigilin transfection was done as in panel (A), but Western analysis was performed with antibodies to ADAR1 or RHA. GAPDH antibodies were used as a negative control.

(C) RHA interacts with the Ku heterodimer. An expression plasmid (from C.G. Lee) for Flag-epitope-tagged RHA was transfected into HEK293T cells. Forty-eight hours after transfection, the cells were lysed and anti-Flag antibodies were used to immunoprecipitate the recombinant RHA. After SDS-PAGE, Western blotting was performed with a mixture of antibodies specific for Ku70 and Ku86.

(D) RNA-dependent protein kinase activity associated with Vigilin. The experiment was carried out as in panel (A), except the immunoprecipitate pellets were incubated with γ-^{32}P-ATP before resolution by SDS-PAGE. The beads were resuspended in 20 μl protein kinase buffer and 10 μl ATP mixture (cold ATP and ^{32}P-ATP). Incubation was for 20 min at 30°C.

(E) A model for the connection between dsRNA editing and heterochromatin.

in the Vigilin complex. HP1 is one attractive possibility. Some nuclear Ku70 colocalizes with HP1, and Ku70 can interact directly with the C-terminal chromo shadow domain of HP1α [30]. Because human HP1α migrates at the same position as the 30 kDa phosphorylated band, we have been unable to directly assess whether this protein is phosphorylated. However, because human HP1α can functionally substitute for the *Drosophila* HP1 protein in insect cells [31] and because HP1 phosphorylation is necessary for its ability to mediate gene silencing [32, 33], we asked whether our complex can phosphorylate added *Drosophila* HP1. This is the case (Figure S3). Finally, histone H2AX is a known target of DNA-PKcs and plays a critical role in chromatin silencing around sites of DNA damage [34]. This protein has also been demonstrated to be a target of phosphorylation in the Vigilin complex (Figure S3).

A Connection between the DNA-Repair Machinery and RNA-Mediated Gene Silencing

DNA damage results in chromatin modifications [35], often with chromatin domains in the megabase range being condensed as the result of dsDNA breaks [36]. The Ku heterodimer is essential for the repair of dsDNA breaks by nonhomologous end joining (NHEJ), but it also has important roles in telomere maintenance and transcriptional regulation [37]. In addition to the reported interaction of human Ku70 with HP1α [30], the *S. cerevisiae* chromatin-silencing factor Sir4 was shown by two-hybrid analysis to interact with yKu70 [38]. Also, the

histone variant H2AX is phosphorylated in response to dsDNA breaks [35] and plays a critical role in the recruitment of repair factors to nuclear foci after DNA damage [34]. These considerations lead us to suggest that the chromatin-silencing machinery involved in NHEJ may also play a role in RNA-mediated gene silencing. The connection between Vigilin and NHEJ is further supported by recent work using a high-throughput RNAi screen to systematically identify synthetic gene interactions in genes involved in the cellular response to dsDNA breaks [39]. Among the newly identified genes whose knockout increased chromosomal nondisjunction was the *C. elegans* Vigilin homolog. Finally, there are several other reports of a possible connection between DNA-repair machinery and RNA-mediated gene silencing [40, 41].

The Biological Consequences of dsRNA in the Nucleus

It is now clear that dsRNA is abundantly expressed in the nuclei of higher eukaryotic cells. Not only is there widespread antisense transcription from genes encoding proteins [42], but it has also become evident that repetitive elements, such as centromeric sequences [43] and arrays of abundant cellular retrotransposons [44], can express significant levels of dsRNA. Therefore, dsRNA is far more prevalent in the nucleus than in the cytoplasm and must be dealt with in ways that are not detrimental to cell function and that do not interfere with the cytoplasmic response pathways.

The RNAi machinery may play an important nuclear role in heterochromatic gene silencing and in chromosome segregation. These effects have been clearly shown in plants [45], in the fission yeast *S. pombe* [3, 4, 46], and recently in *Drosophila* [6] and vertebrate cells [8]. However, most higher-eukaryotic cells express ADAR in their nuclei [47], and editing is incompatible with the activity of the Dicer enzyme [48]. Therefore, in these cells the editing machinery might provide a mechanistic alternative to the RNAi machinery. Like the RNAi machinery, Vigilin has now been implicated in mRNA stability and translation in the cytoplasm as well as in chromosome segregation and heterochromatin formation in the nucleus. Whether the RNAi and Vigilin pathways for heterochromatin formation are connected to each other biochemically is not yet clear. It is possible that in lower organisms, where the RNAi response is more robust, this machinery might be more important in gene silencing in the nucleus. In higher organisms, where editing in the nucleus is known to be efficient, an ADAR-related pathway might predominate. On the other hand, these two pathways may intersect or compete in ways that could allow more subtle regulation of gene silencing. Consistent with this, ADAR levels have been shown to affect transgene-induced gene silencing in *C. elegans* [49], and some, but not all, RNAi-defective mutants in this organism lead to the mobilization of transposons [50]. Also, evidence has been found for editing of transposon transcripts [51]. Thus, it remains possible that the Vigilin system is responsible for some transposon silencing, which may not completely overlap with the silencing pattern characteristic of the RNAi machinery.

A Model for a Connection between Editing and Gene Silencing

Although the results we have presented are consistent with a role of Vigilin in mediating heterochromatic gene silencing as the result of ADAR editing of dsRNAs (Figure 4E), other interpretations remain possible. The RNAi and editing machineries may act independently or in concert to allow the establishment of heterochromatin. Alternatively, they may compete with or antagonize each other. ADAR editing within RNA duplex structures is not completely random. ADAR preferentially deaminates adenosines whose 5′ neighbors are either adenosines or uridines [52]. Thus, in many nuclear dsRNAs, and especially in those formed from simple repetitive sequences, editing may be less efficient than in more complex dsRNAs or may leave long regions relatively unedited. ADAR editing may thus limit the target availability for the RNAi machinery, perhaps by directing it to the most appropriate sequences and making it more specific.

Supplemental Data

Three additional figures and a table are available with this article online at http://www.current-biology.com/cgi/content/full/15/4/384/DC1/.

Acknowledgments

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